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(54) Title: A PROCESS FOR PREPARING RIPE PROTEINS FROM FUSION PROTEINS, SYNTHETIZED IN PRO- OR EUKARYOTIC CELLS

(57) Abstract

A fusion protein with the amino acid sequence: $(Y_m...Y_2\text{-}Y_1)\text{-}(Pro)_p\text{-}(X_1\text{-}X_2...X_n)$ in which $(Y_m...Y_2\text{-}Y_1)\text{-}(Pro)_p$ is the pro-sequence and the rest is the ripe protein, m is an integer greater than 2, and Y is an arbitrary amino acid, p is O if X_1 or X_2 is Pro, and 1 if X_1 or X_2 is different from Pro, X is an arbitrary amino acid, and n is integer equal to or greater than 4, is converted into ripe proteins by enzymatic cleavage, which is carried out by stepwise cleavage off of the amino acid groups $Y_m...Y_2$ if $p = 1$ or $X_1 =$ Pro, or the groups $Y_m...Y_2\text{-}Y_1$ if $X_2 =$ Pro, by means of an aminopeptidase, and then the two amino acids Y_1-Pro if $P = 1$ are cleaved off enzymatically in one or two steps in a manner known per se, and similarly Y_1 alone is cleaved off if $X_1 =$ Pro. The preferred aminopeptidase is leucine aminopeptidase. The process enables removal in vitro of the formyl methionine or the methionine group and optionally the pro-sequence from a protein synthetized in pro- or eukaryotic cells by genetic engineering, and thus achievement of a desired ripe protein, such as human growth hormone or human proinsulin.

The basic molecular-biological principles which, based on DNA, lead to the formation of a protein are well-known to those skilled in the art.

When proteins are synthetized in cells, the product first formed is seldom equal to the final, ripe protein. Most often, e.g. a somewhat longer amino acid chain is synthetized which is then cut to the length of the desired ripe protein, probably enzymatically. The cut amino acid sequences are usually disposed in the N-terminal end of the protein, and are called pro-sequences or signal sequences, according to their function and time of cleavage. Usually, only the ripe protein has full biological activity.

Initiation of the biosynthesis of proteins/peptides takes place by a mechanism causing the N-terminal amino acid in the produced protein or peptide to always be menthionine (in eukaryotic cells) or N-formyl methionine (in bacteria). This N-terminal methionine (or formylmethionine) is often included in a pro-sequence or a signal sequence, which is cleaved later, and therefore is not present in the ripe protein.

Discoveries and progress in recent years within molecular biology have made it possible to transfer genes (DNA segments) <u>in vitro</u> from one organism to another, unrelated organism. Thus, e.g. human genes can be transferred to bacteria, such as E. coli. If the human DNA segment is modified so as to resemble bacterial DNA, the bacteria may in certain cases be caused to synthetize a protein identical or closely related to the protein which is coded for by the human DNA segment. Normally, however, great problems are associated with making bacteria synthetize a product by this genetic

engineering which is structurally identical to the desired ripe eukaryotic protein. Usually, the resulting bacterial product is a fusion protein between an amino acid sequence of a bacterial protein and the desired eukaryotic protein or it is the eukaryotic protein provided with an N-terminal formyl methionine group. These products are only rarely further modified in bacteria.

To remedy this situation, it is proposed in the DK patent application No. 1616/81 to couple a bacterial signal sequence directly to a eukaryotic protein, and the bacteria itself will hopefully then cleave off the signal sequence. However, this is not a universally useful method because the cleavage also seems to depend upon the amino acid sequence of the eukaryotic protein.

Further, it is proposed in the DK patent application No. 888/81 to incorporate specific proteolytic or chemical cleavage sites at the border between the bacterial and the eukaryotic part of the protein. The usefulness of this method, however, is limited because the desired ripe eukaryotic protein must not contain the same proteolytic or chemical cleavage site.

The fusion protein may be cleaved chemically at specific amino acids (e.g. after methionine using BrCN). However, this method requires that the specific amino acid in question is not also present in the ripe protein, and the method is vitiated by a certain risk due to the toxicity of BrCN.

When synthetized in bacteria, the newly synthetized protein always contains, as mentioned, N-terminal N-formyl methionine and optionally a pro-sequence which

is not present in proteins of eukaryotic cells and must therefore always be removed if the bacterially synthetized protein is to have the same primary amino acid sequence as the desired protein from e.g. humans.

Similarly, proteins formed in eukaryotic cells always contain N-terminal methionine.

In addition, a suitable DNA sequence may be incorporated by genetic engineering in front of the DNA segment coding for the desired protein so that the expressed fusion protein is caused to contain an appropriate N-terminal amino acid sequence. This amino acid sequence may e.g. be selected so that the purification of the fusion protein will be easier, that a desired proteolytic cleavage site results or that the fusion protein is transported out of the bacteria. Such an N-terminal amino acid sequence must of course also be removed to provide the desired, ripe protein.

Generally, it may be said that when the bacteria cannot be caused to produce the desired protein directly, then the resulting fusion protein must be treated enzymatically or chemically so that the additional N-terminal amino acids are removed without the reactions influencing the desired protein.

A process for enzymatic cleavage of a fusion protein is known from the EP Publication No. 0020290. In this process the enzyme collagenase is used for cleaving a bond in the signal or pro-sequence of the fusion protein, viz. the bond Xyz-Gly in which Xyz is a peptide chain incorporated in the pro-sequence. The remaining amino acid or acids in the pro-sequenc are then removed enzymatically.

However, it has been found that collagenase and other endopeptidases are deficient in specificity, see Biochim. Biophys. Acta, 271 (1972) 133-144. Moreover, the collagenases are active only in connection with proteins of a specific steric structure, see Peptides 1980, Proceedings of the sixteenth European Peptide Symposium, Ed. K. Brunfeldt (Scriptor Copenhagen 1981).

Proteolytic enzymes (proteases) are characterized in that in certain circumstances they can hydrolyze the peptide bonds between the amino acids in proteins (and peptides). The proteolytic enzymes constitute a very large and heterogenous group of proteins, both as regards mode of operation and the specificity with which the amino acid sequences are cleaved. In brief summary, the proteolytic enzymes are divided into two main groups: exo- and endoproteases. As indicated by the names, exoproteases cleave off amino acids from the end of the protein chain, whereas endoproteases can directly cleave in the interior of a protein. Aminopeptidases are exoproteases cleaving off amino acids from the N-terminal end of the protein chain. Proteases often have a high degree of specificity as they mainly cleave bonds in specific amino acid sequences. The cleavage is not only dependent upon the amino acid sequence, but also the sterical structure of the substrate may have a great influence on the cleavage rate. The ability of proteases to cleave proteins in a specific manner has been used for a number of years for characterization, analysis and modification of proteins.

The present invention is based on the use of an exopeptidase which cleaves off the amino acid groups in the pro-sequence of the fusion protein one by one until only the group Y-Pro remains in which Y is an arbitrary

amino acid.

The exopeptidase used is an aminopeptidase, and as such leucine aminopeptidase is preferred.

The object of the present invention is to provide a process in which any protein, synthetized by genetic engineering in pro- or eukaryotic cells, can be converted *in vitro* by enzymatic processes to a protein having an amino acid sequence identical to the selected eukaryotic protein, and thus solve the following two problems in the preparation of ripe proteins:

a) removal of the formyl methionine or the methionine group,

b) release of the ripe protein from the pro-sequence.

According to the invention, proteases are used, and in particular aminopeptidases, for cleaving off, if necessary, formyl methionine and optionally other N-terminal amino acids in a synthetized fusion protein.

Thus, the invention relates to a process for preparing ripe proteins by enzymatic cleavage of a fusion protein with the amino acid sequence:

$$(Y_m...Y_2-Y_1)-(Pro)_p-(X_1-X_2...X_n)$$

in which $(Y_m...Y_2-Y_1)-(Pro)_p$ is the pro-sequence and the rest is the ripe protein, m is an integer greater than 2, and Y is an arbitrary amino acid, P is 0 if X_1 or X_2 is Pro, and 1 if X_1 or X_2 is different from Pro, X is an arbitrary amino acid, and n is an integer equal to or greater than 4, and the process is characterized by carrying out the enzymatic cleavage by stepwise

cleavage off of the amino acid groups $Y_m...Y_2$ if p = 1 or X_1 = Pro, or the groups $Y_m...Y_2-Y_1$ if X_2 = Pro, by means of an aminopeptidase, and then the two amino acids Y_1-Pro if p = 1 are cleaved off enzymatically in one or two steps in a manner known per se, and similarly, Y_1 alone is cleaved off if X_1 = Pro.

According to the invention, fusion proteins as stated in step (i), which do not contain a formyl methionine group, can be obtained i.a. in one of the following ways:

1) The DNA coding sequence of a suitable prokaryotic N-terminal pro-sequence containing or constituting a signal sequence can be spliced on to the coding sequence of the desired protein at gene level. The DNA sequence corresponding to this pro-sequence may be selected from among the large number of naturally occurring sequences or may be synthetized <u>in vitro</u> when their structure at nucleotide and amino acid level is known. After the synthesis of the fusion protein in bacteria, the signal sequence (containing the N-terminal f-Met.) is cleaved off by the bacteria <u>in vivo</u>.

2) The DNA coding sequence of the selected pro-sequence is coupled to the coding sequence of the desired protein. The fusion protein (with the N-terminal formyl methionine) synthetized in bacteria, is isolated completely or partly from the cells or the bacterial supernatant. The fusion protein is then treated with aminopeptidase I (AP I) (E.C. 3.4.11.12) from Bacillus stearothermophilus (Roncari & Zuber, Int. J. Protein Research II, 191 (1970)). This aminopeptidase has the same broad specificity as most of the other aminopeptidases, but is moreover able to

cleave off N-formyl methionine in the above-mentioned fusion product. This cleavage reaction is to be optimized with respect to time and enzyme concentration as, in case of prolonged incubation, aminopeptidase I can also hydrolyze amino acids of the desired product. This problem can be reduced by incorporating one or more of the amino acid sequences Gly-Ala, Gly-Gly or Lys-Leu in the pro-sequence because these peptide bonds are hydrolyzed only slowly by the above-mentioned aminopeptidase I.

3) The DNA coding sequence of the pro-sequence is so formed that the pro-sequence is caused to contain highly active cleavage sites for trypsin (E.C. 3.4.21.4.) or trypsin-like proteases. Such cleavage sites may e.g. consist of the amino acid sequences Arg-Arg, Lys-Lys, Lys-Arg and/or Arg-Lys. After the synthesis of the fusion protein it is isolated <u>in vivo</u> wholly or partly, and is treated <u>in vitro</u> with trypsin or the trypsin-like protease.

In each of the three above-mentioned procedures it is thus possible according to the invention to provide a protein containing the amino acid sequence corresponding to the desired protein as well as an N-terminal pro-sequence without formyl methionine. In this pro-sequence the C-terminal amino acid, which is bonded directly to the N-terminal amino acid in the desired protein, must be proline, unless the desired protein itself contains proline as N-terminal or next-to-the-outermost N-terminal amino acid. The pro-sequence may then be removed specifically <u>in vitro</u> by treating the fusion protein with a suitable aminopeptidase, e.g. leucine aminopeptidase (E.C. 3.4.11.1). This commercially available aminopeptidase will cleave the sequence, but will stop after having hydrolyzed the bond just before the dipeptide

X-Pro. After removal or inactivation of the leucine aminopeptidase, the resulting remaining fusion protein can be treated in one of the following ways if X and optionally Pro are to be cleaved off:

a) X is different from Gly.
First the amino acid X is cleaved off, e.g. with aminopeptidase P (3.4.11.9) or with prolidase, and then, if desired, proline is cleaved off with proline iminopeptidase (3.4.11.5).

b) X = Gly.
Here Gly-Pro can be cleaved off in the same manner as mentioned under point a). However, when e.g. a Gly-Pro hydrolyzing dipeptidylpeptidase IV (3.4.14.-) is used, whole dipeptides Gly-Pro can be cleaved off in one step.

EXAMPLE 1

A cloned DNA sequence coding for a protein with an amino acid sequence as in human growth hormone, hGH, (191 amino acid residues of which the first four amino acids are Phe-Pro-Thr-Ile) is coupled with the following synthetically produced, dual-stranded DNA sequence so that the 3' end of the +strand is coupled to the +5' end of the above-mentioned gene, and the 5' end of the -strand of the synthetic DNA sequence is coupled to the 3' end of the above-mentioned gene by blunt end ligature.

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+ 5' GATCCATGCTGGCTGTAAGC 3'
- 3'      TACGACCGACATTCG 5'
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where the 5 first nucleotides in the +strand are part of a Bam Hl restriction site, and the following nucleotide sequences code for the amino acids Met, Leu, Ala, Val, Ser.

The above-mentioned gene is introduced by ordinary gene cloning techniques into an expression plasmide containing a fusioned Trp-Lac promotor as well as the SD sequence AGGA; this structure should therefore express Met-Leu-Ala-Val-Ser-hGH.

This plasmide structure is then introduced into an E. coli cell by prior art techniques. A suitable clone containing the above-mentioned structure is isolated and cultivated in a 30 l scale. The cells are harvested by centrifugation and are suspended in a small volume and lyzated using a so-called "French press".

The expected fusion protein could be demonstrated in the above-mentioned bacterial extract by immunological methods using hGH antibodies.

The fusion product was purified from this extract by hydrophobic interaction chromatography, ammonium sulfate precipitation, gel filtration and anion exchange. Fractions containing the fusion protein were determined immunologically using hGH antibodies.

The purified fusion protein was evaluated to be more than 98% pure by polyacrylic amide gel electrophoresis and ion exchange HPLC. 15 mg were isolated from a 30 l culture. Possible disulfide bridges in the purified fusion protein were reduced and S-carbamidomethylated in principle as described by L. Graf et al.: FEBS Letters 66(2), 233 (1976). An N-terminal sequence determination according to Edman of the purified and reduced and S-carbamidomethylated fusion protein showed the expected amino acid sequence Met-Leu-Ala-Val-.

This reduced and S-carbamidomethylated product was then treated with leucine aminopeptidase (EC 3.4.11.1) as

described by D.H. Sprekman et al., J. Biol. Chem. 212, 255 (1955) and A. Light: In: Methods in Enzymology, vol. XI, Ed.: C.H.W. Hirs (1967), Academic Press, p. 426. However, the enzymatic hydrolysis was carried out in the presence of urea and aprotinin.

The the reaction mixture was fractionated by ion exchange, and protein from fractions containing hGH immunoreactivity was isolated. Evaluated by polyacrylic amide gel electrophoresis and ion exchange HPLC the protein was more than 98% pure. N-terminal determination according to Edman showed Phe as the main component.

EXAMPLE 2

Production of human proinsulin

A gene coding for a protein with an amino acid sequence as in human proinsulin (86 amino acid residues of which the first 4 amino acids are Phe-Val-Asn-Gln), is coupled with the following synthetically produced, dual-stranded DNA sequence so that the 3' end of the +strand is coupled to the 5' end of the +strand of the above-mentioned gene, and the 5' end of the -strand of the synthetic DNA sequence is coupled to the 3' end of the above-mentioned gene by blunt end ligature.

			Met	Leu	Val	Ala	Gly	Pro	
+	5'	ATTC	ATG	TTG	GTT	GCT	GGT	CCA	3'
-		G	TAC	AAC	CAA	CGA	CCA	GGT	

The 5 first nucleotides in the +strand are part of an Eco RI restriction site, and the subsequent nucleotide sequences code for the amino acids Met-Leu-Val-Ala-Gly-Pro.

The above-mentioned gene is introduced by ordinary gene cloning techniques into an expression plasmide containing the promotor for 3-phosphoglycerate kinase and the terminator for the FLP gene, both from S.cerevisiae. (Ref.: Hitzeman et al. (1983), Science 219 620-625).

This plasmide structure is then introduced into an S.cerevisiae cell by prior art techniques. An S.cerevisiae clone containing the above-mentioned structure is isolated and cultivated in a suitable volume. The yeast cells are harvested and lyzated using a so-called "French press".

A protein assumed to be the expected fusion protein is demonstrated in the extract by immunological methods using proinsulin antibodies.

The fusion protein is purified by hydrophobic interaction chromatography.

The fusion protein is purified to >90% purity, evaluated by polyacrylic amide electrophoresis and ion-exchange HPLC. An N-terminal sequence determination according to Edman is carried out to confirm the expected amino acid sequence (corresponding to Met-Leu-Val-Ala-Gly-Pro-Phe ---).

This purified product is treated with leucine amino-peptidase (EC 3.4.11.1.) as described by D.H. Sprekman et al., J. Biol. Chem. 212, 225 (1955) and A. Light: In: Methods in Enzymology, vol. VI, Ed.: C.H.W. Hirs (1967), Academic Press, p. 426. Then the reaction mixture is fractionated by ion xchange, and protein from fractions containing proinsulin immunoreactivity is isolated. The purification is better than 90%

evaluated by polyacrylic amide electrophoresis and ion-exchange HPLC. Gly-Pro-Phe is found in the isolated product by three consecutive N-terminal degradations according to Edman.

Gly-Pro is removed enzymatically from this product in a manner known per se in a 1-step reaction using post-proline dipeptidyl aminopeptidase (EC 3.4.14.-), cf. R. Walter, W.H. Simmons & T. Yoshimoto: Molecular & Cellular Biochemistry 30(2) (1980), 111-127. Or, alternatively, in a 2-step process, cf. R.I. Delange & E.L. Smith, in: The Enzymes. Ed.: P.D. Boyer, Academic Press (1971), p. 115, to produce human proinsulin.

EXAMPLE 3

An 0.1 M Tris buffer is used in which pH is adjusted to 8.5 with 1 M hydrochloric acid. This buffer is admixed with $MnCl_2$, $4H_2O$ so that the concentration of Mn^{2+} is 0.5 mM. Leucine aminopeptidase (Sigma) is incubated at 37°C for 45 minutes in the above-mentioned buffer (1:9). 950 nmoles~1 mg Gly-Phe-Phe-Tyr-Thr-Pro-Lys-(Ala) is dissolved in 2 ml of the above-mentioned buffer. This mixture is admixed with 1 ml of the incubated enzyme solution, 22 units leucine aminopeptidase. The mixture is left to stand at 37°C. After 30, 60, 90 and 120 minutes 300 µl (~9.5 nmoles) are removed, and the reaction is stopped by the addition of 50 µl EDTA (12 mg/ml) and 10 µl concentrated hydrochloric acid. Amino acid analysis is carried out on 100 µl (corresponding to 26.4 µmoles) without preceding acid hydrolysis. The result of the analysis is in nmole: Gly: 28.7, Tyr: 22.6 and Thr: 43.9. Thr could not be demonstrated by the amino acid analysis.

EXAMPLE 4

0.8 mg (1.0 µmole) Ile-Ser-Pro-Ser-Arg-Ser-Gln is dissolved in 2 ml of the buffer described in example 1. 1 ml (22 units) of incubated leucine aminopeptidase is added, like in example 1. The mixture is left to stand at 37°C. After 30, 60, 90 and 120 minutes, 300 µl (~100 nmoles) are removed. The reaction is stopped as described in example 1. Amino acid analysis is carried out on 100 µl (corresponding to 27,78 nmoles) without preceding analysis. 18 nmoles Ile are seen in all the samples.

EXAMPLE 5

A synthetic peptide 0.9 mg (93 nmoles) Tyr-Pro-Ala-Gln-Ile-Lys-Val is treated with leucine aminopeptidase as described in example 1. 300 µl are removed after 30, 60 and 240 minutes. Amino acid analysis is carried out on 100 µl (corresponding to 25.8 nmoles) without preceding hydrolysis. Between 0 and 11 nmoles of Thr or Ser, Glu, Ala, Val, Ile and Tyr could be detected. This is probably present because the synthetic peptide was not quite pure and a proline is presumably missing.

P a t e n t c l a i m s:

1. A process for preparing ripe proteins by enzymatic cleavage of a fusion protein with the amino acid sequence:

$$(Y_m \ldots Y_2\text{-}Y_1)\text{-}(Pro)_p\text{-}(X_1\text{-}X_2 \ldots X_n)$$

in which $(Y_m \ldots Y_2\text{-}Y_1)\text{-}(Pro)_p$ is the pro-sequence and the rest is the ripe protein, m is an integer greater than 2, and Y is an arbitrary amino acid, P is 0 if X_1 or X_2 is Pro, and 1 if X_1 or X_2 is different from Pro, X is an arbitrary amino acid, and n is an integer equal to or greater than 4,
c h a r a c t e r i z e d by carrying out the enzymatic cleavage by stepwise cleavage off of the amino acid groups $Y_m \ldots Y_2$ if p = 1 or X_1 = Pro, or the groups $Y_m \ldots Y_2\text{-}Y_1$ if X_2 = Pro, by means of an aminopeptidase, and then the two amino acids Y_1-Pro if p = 1 are cleaved off enzymatically in one or two steps in a manner known per se, and similarly Y_1 alone is cleaved off if X_1 = Pro.

2. A process according to claim 1,
c h a r a c t e r i z e d in that the aminopeptidase is leucine aminopeptidase.

3. A process according to claim 1 or 2,
c h a r a c t e r i z e d in that the fusion protein includes the amino acid sequence occurring in a human growth hormone.

4. A process according to claim 1 or 2, c h a r a c t e r i z e d in that the fusion protein contains proinsulin.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK83/00118

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 P 21/06

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC 1+2	C 12 D 13/06
IPC 3	C 12 P 21/00,02,06; C 12 N 9/48, 15/00
US Cl	435:68, 69, 70, 172, 212, 317; 195:4, 29

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,P	EP, A2, 0 089 626 (G D SEARLE & CO) 28 September 1983 especially p 6-9, 18-19 & JP, 58216697	1, 3-4
Y	Hirs, C H W (Ed), Methods in Enzymology, Vol 6, 1967, Academic Press Inc., New York, p 426-36	1-2
Y,P	Chemical Abstracts, Vol 98 (1983), abstract No 212 112, Biochim. Biophys. Acta 1983, 743(3), 437-47 (Eng)	1
A	EP, A1, 0 020 290 (SCHERING AKTIENGESELL-SCHAFT) 10 December 1980	1, 3-4

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
1984-02-20	1984 -02- 28
International Searching Authority [1]	**Signature of Authorized Officer** [20]
Swedish Patent Office	Margareta Danielsson Margareta Danielsson

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